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Distribution of Lactate Dehydrogenase (and its E-isozymes) in the developing and adult Retina of the Guppy *(Lebistes reticulatus)* [1]

by

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with 19 figures

The enzyme Lactate Dehydrogenase (LDH) occurs in mammalian tissues in five distinct isozymes. They can be separated by electrophoresis and stained with histochemical methods, and appear as blue bands due to the reduction of a tetrazolium salt to formazan. The relative intensity of the different bands thus obtained, differs considerably from tissue to tissue. In the heart the most anodic bands predominate, whereas in the skeletal muscle the most cathodic bands are more pronounced. It has been shown that in mammals each of the five isozymes is a tetramer composed of two subunits, called A and B, encoded by two different genes. In addition, a third subunit, C, is present in the testis of several mammals and birds. (Batellino *et al.*, 1968).

The LDH isozyme patterns of fish show great variation. From one to 18 different isozymes have been found in some thirty species tested (Markert and Faulhaber, 1965; Massaro and Markert, 1968). One set of isozymes, with extreme anodal electrophoretic mobility, has been observed in the eye and brain only of many teleosts (E-isozymes) (fig. 1). It has been suggested by various authors, that these E-isozymes reside in the retina, but apart from the work of Whitt and Booth (1970) the teleostean retina has not been subjected to histo-

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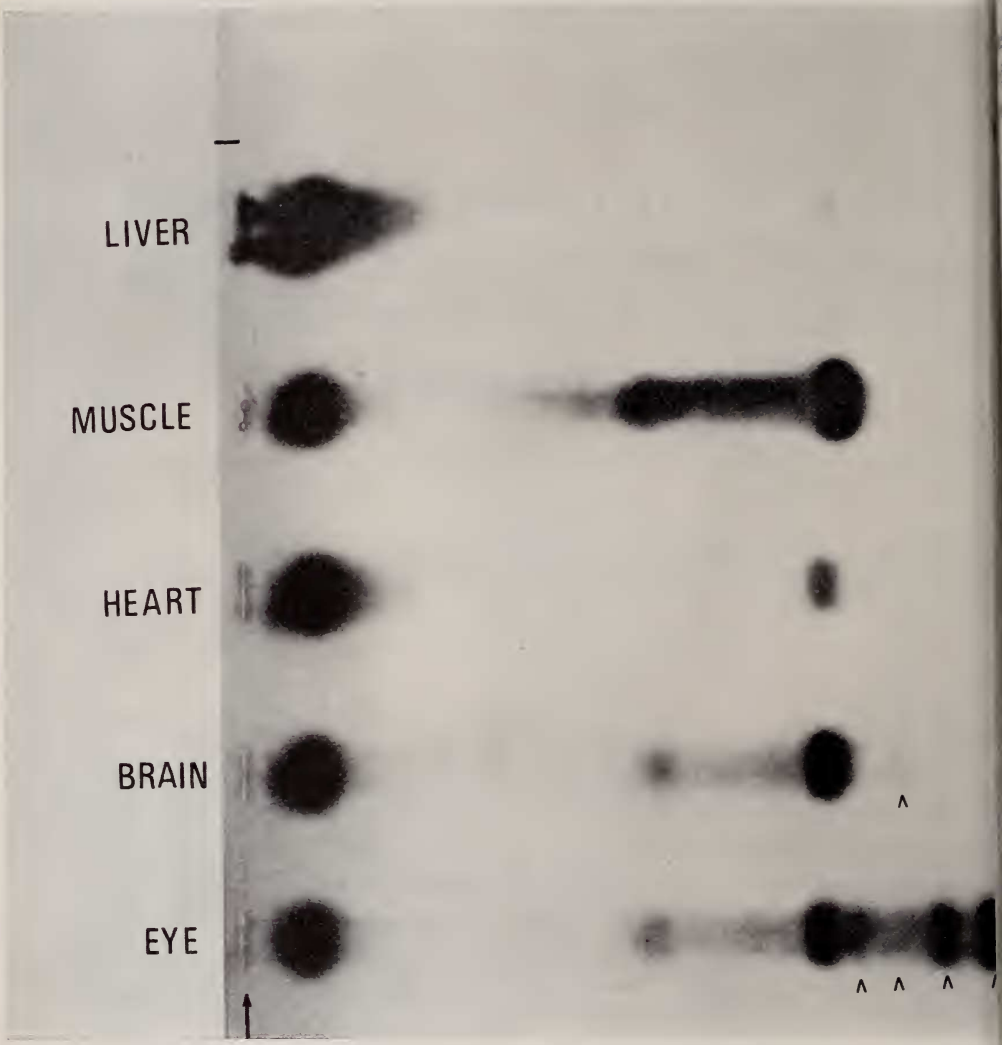


FIG. 1.

Lactate dehydrogenase isozyme pattern of different organs of adult *Lebistes*.
(Incubation mixture: 204.5 mg of DPN (Diphosphopyridine nucleotide), 54.5 mg of nitroblue-tetrazolium 5.5 mg of PMS (Phenazine methosulphate), 10.2 ml of neutralized lactic acid. Bidistilled water added to final volume of 100 ml; incubation time: 30 min. at 37° C).
∧ indicate E-bands, unique to eye and brain.
↑ denotes point of application of sample.

chemical analysis. It was also proposed by different workers (including the author) that the first appearance of the E-isozymes during ontogeny is connected with the onset of visual function, but no histochemical evidence to substantiate this has been brought forward so far.

The purpose of the following investigations was: 1) to establish with starch gel electrophoresis at what developmental stage of the embryo or postembryo of *Lebistes* the E-bands appear; 2) to determine with kryostat sections in what layers of the retina they reside and then to identify their subcellular location;

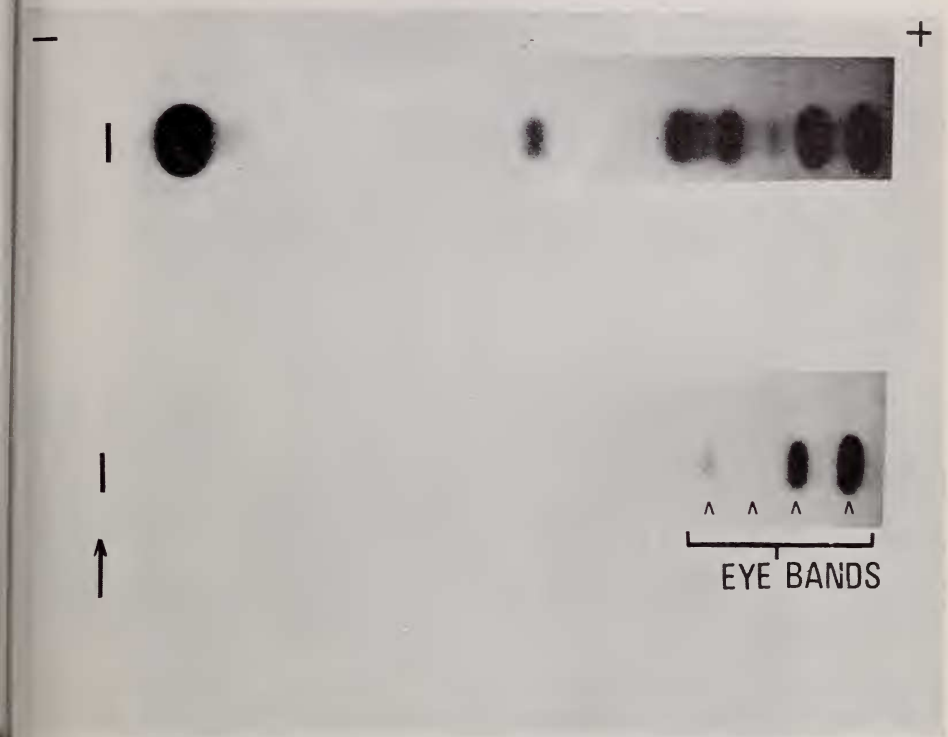


FIG. 2.

Effect of inhibitor (3 M urea) on lactate dehydrogenase isozyme pattern of adult *Lebistes* eye. Starch gel plate sliced horizontally: upper half incubated without, lower half with urea.

3) to follow the development of the E-isozymes both with starch gel electrophoresis and kryostat sections until sexual maturity is reached.

RESULTS

The first problem was to show the E-isozymes selectively. To that effect several known inhibitors for mammalian LDH isozymes were tested. The only one that had an effect on starch gel, as well as on kryostat sections, was urea in a 3 M concentration (fig. 2).

For the purpose of clarity the results obtained with the adult eye are presented first. The overall structure of the *Lebistes* eye is given in fig. 3.

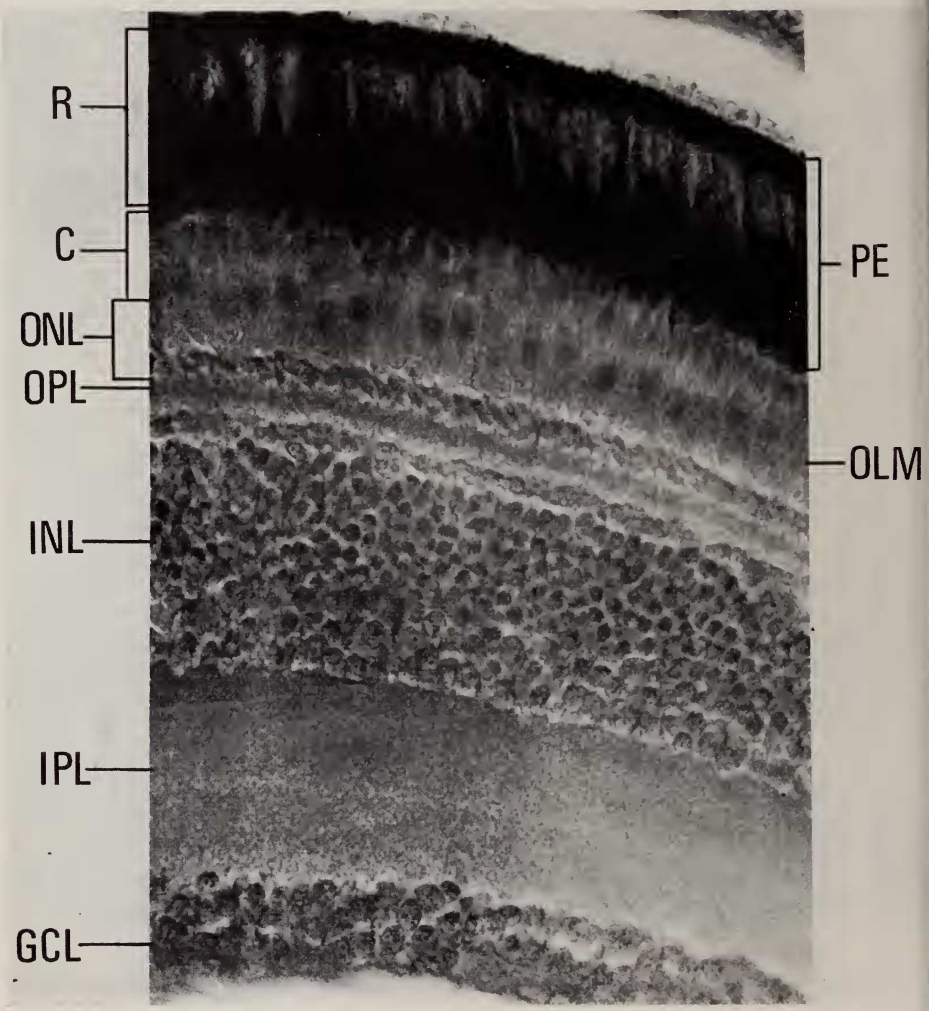


FIG. 3.

Photomicrograph of *Lebistes* retina to show the different layers (azan stain). Designation of layers see p. 775.

Adult Eye :

The adult eye stained for LDH indicates clearly that most of the activity resides in the photoreceptor layer (Fig. 4). Sections of the same eye, but treated with the inhibitor, reveal the E-isozymes in the same location. The inner layers

of the retina do not stain at all, or at most very faintly. Also remnants of skin and eye muscles, adhering to the eye, did not take up the stain which indicates that inhibition is effective (fig. 5).

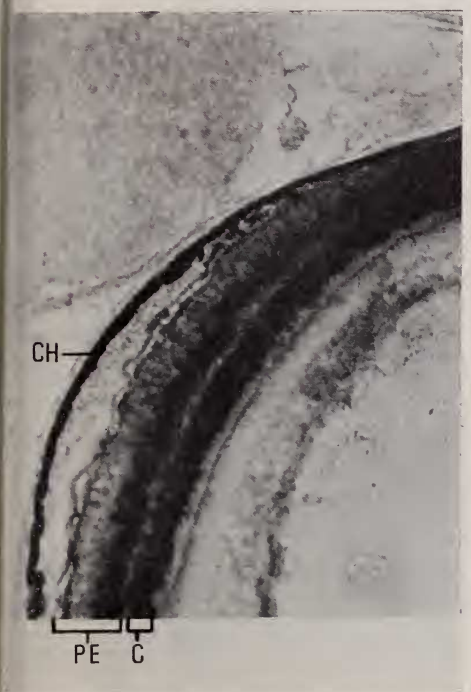


FIG. 4.

Photomicrograph of *Lebistes* retina (kryostat section) stained for lactate dehydrogenase activity (Incubation medium: 30 mg DPN, 15 mg nitroblue tetrazolium, 10 mg PMS, 3 ml sodium lactate (0.05 M), 8 ml phosphate buffer (0.1 M, pH 8.4). Distilled water added to final volume of 30 ml. Incubation time: 15 min. at 37° C).

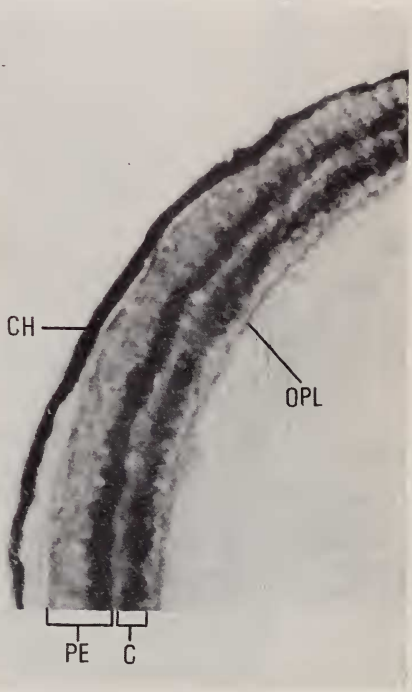


FIG. 5.

Photomicrograph of *Lebistes* retina (kryostat section) stained for E-isozyme activity. (Incubation mixture: same as for fig. 4, but 3 M urea added.)

The photoreceptors of *Lebistes* show a very complex arrangement. Three different kinds of cones are present—inner, middle and outer—the last being twin cones. The cones are arranged in a regular mosaic pattern, whereas the rods are interspersed at random (MÜLLER, 1952). A diagram of a generalized photoreceptor is shown in fig. 6. The relative positions of rods and cones in dark and light adapted retina of *Lebistes* are given in figs. 7 and 8.

When the kryostat sections already referred to, are viewed with oil immersion, it is evident that the LDH activity is most pronounced in the inner segments (ellipsoids) of all three types of cones. In the light adapted eye, the outer segments

appear clearly stained also, whereas in the dark adapted eye they are not visible. This may be because the rods are superimposed. The inner segments of the rods show very faint LDH activity in the dark adapted eye; they cannot be recognized in the light adapted eye since they are covered by pigment. The outer segments of the rods in both dark and light adapted eye show a weak pink staining reaction in the inner half and blue granules in the outer half (figs. 9 and 10). The cytoplasm in the outer nuclear layer and the outer plexiform layer, stains uniformly but moderately (figs. 9 and 10).

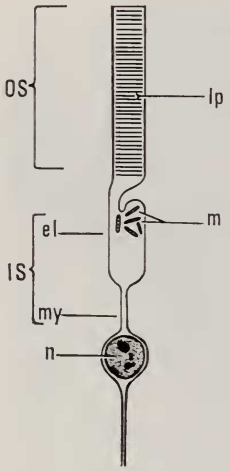


FIG. 6.

Diagram of generalized photoreceptor
el = ellipsoid, m = mitochondria, my = myoid, n = nucleus, lp = lipoprotein lamellae (sacs).

The effectiveness of nitroblue tetrazolium in localizing LDH at the subcellular level in the light adapted eye has been checked by varying the temperature, the pH and the constituents of the incubating medium and by pretreating the sections with acetone. The pink precipitate in the outer segments of the rods proves to be " nothing dehydrogenase ", and enzymic in nature, whereas the blue droplets seem to be due to the accumulation of formazan deposits by lipid granules (fig. 12). The outer and inner segments of all three types of cones stain under all conditions, except when incubated at 4°C (normal temp. 37°C), and when lactate is omitted.

FIG. 7.

Diagram of dark adapted retina of *Lebistes* (outer and inner segments (ellipsoids) only).

FIG. 8.

Diagram of light adapted retina (outer and inner segments (ellipsoids) only).

FIG. 9.

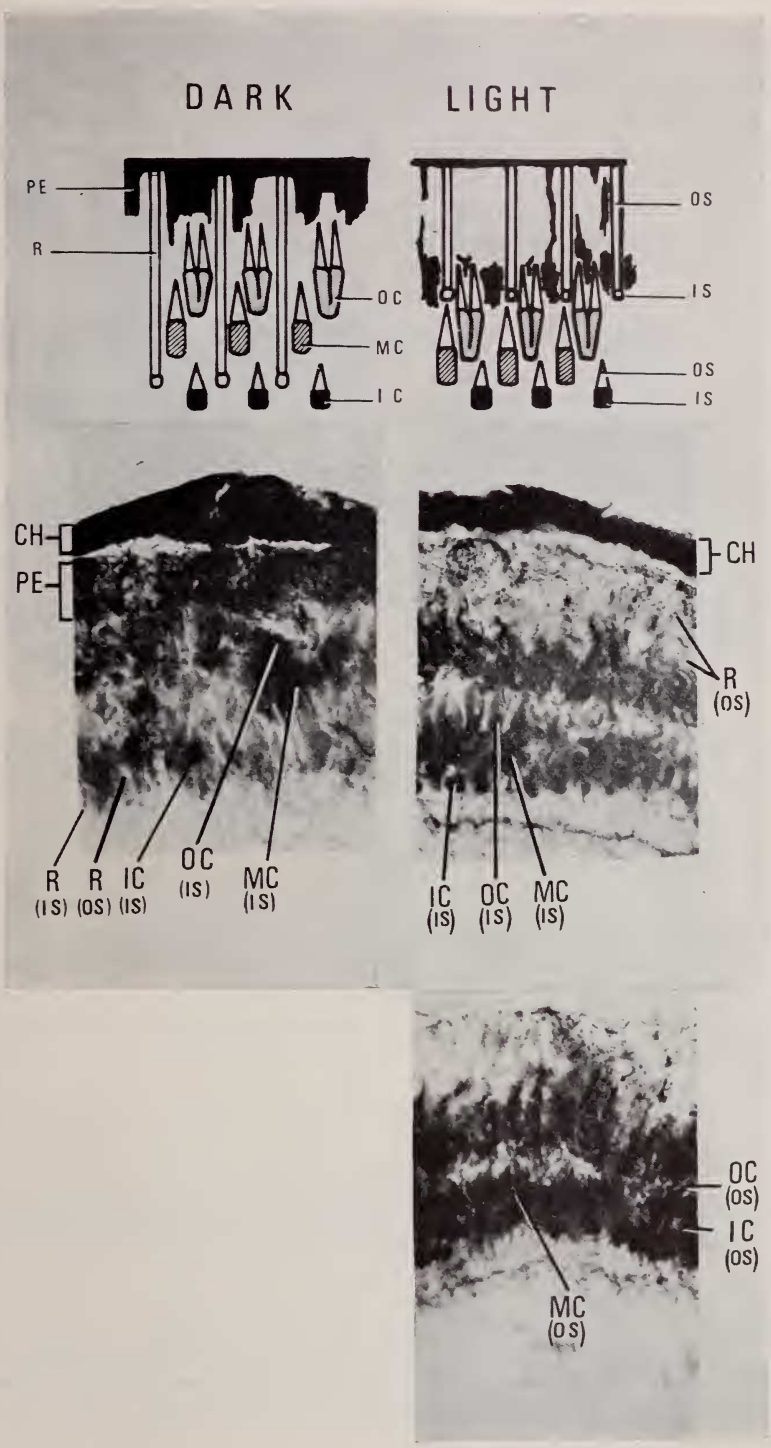
Kryostat section of dark adapted adult retina, stained for lactate dehydrogenase activity (viewed with oil immersion).

FIG. 10.

Kryostat section of light adapted adult retina, stained for lactate dehydrogenase activity. Detail of fig. 4.

FIG. 11.

Kryostat section of light adapted adult retina, stained for E-isozymes. Detail of fig. 5.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Thus, it is concluded that the formazan deposits in the outer and inner segments of all three types of cones represent LDH activity. The outer segments of the rods, however, are devoid of this enzyme. The " nothing dehydrogenase " observed in this region is probably alcohol dehydrogenase, an enzyme engaged in the visual process. As previously mentioned, the inner segments of the rods cannot be seen with certainty since pigment is accumulated in the region.

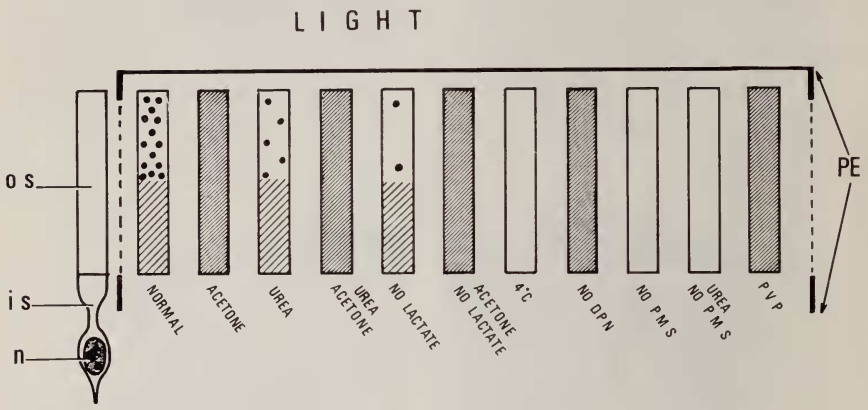

FIG. 12.

Outer segments of rods (adult, light adapted eye of *Lebistes*) Kryostat sections, subjected to variations in the staining method.

Normal = Incubation mixture as for fig. 4.
Normal = Incubation mixture as for fig. 4.
Acetone = acetone treatment prior to incubation.
Urea = 3 M urea added to incubation mixture.
No lactate = lactate omitted from incubation mixture.
4° C = incubation at 4° C (instead of 37° C).
No DPN = β-Diphosphopyridine nucleotide omitted from incubation mixture.
No PMS = Phenazine methosulphate omitted from incubation mixture.
PVP = Polyvinylpyrrolidone added to incubation mixture.

= blue granules.

= light pink stain.

= strong pink stain.

Inspection with oil immersion of sections treated with the inhibitor, revealed the very same picture as given above for total LDH activity, with the only difference that the staining of inner and outer segments of cones is less intense (fig. 11).

Developing eye :

Only results of the light adapted eye are reported. *Lebistes* is viviparous; the embryonic period lasts 30 days at 22°C. The different embryonic stages have been described previously (KUNZ, 1963, 1964, 1971).

The first appearance of the E-bands on starch gel is observed in an embryo of 20 days (stage 7). They show, already, adult distribution of intensity; they are, however, much weaker (fig. 13). Histological staining of the eye reveals that

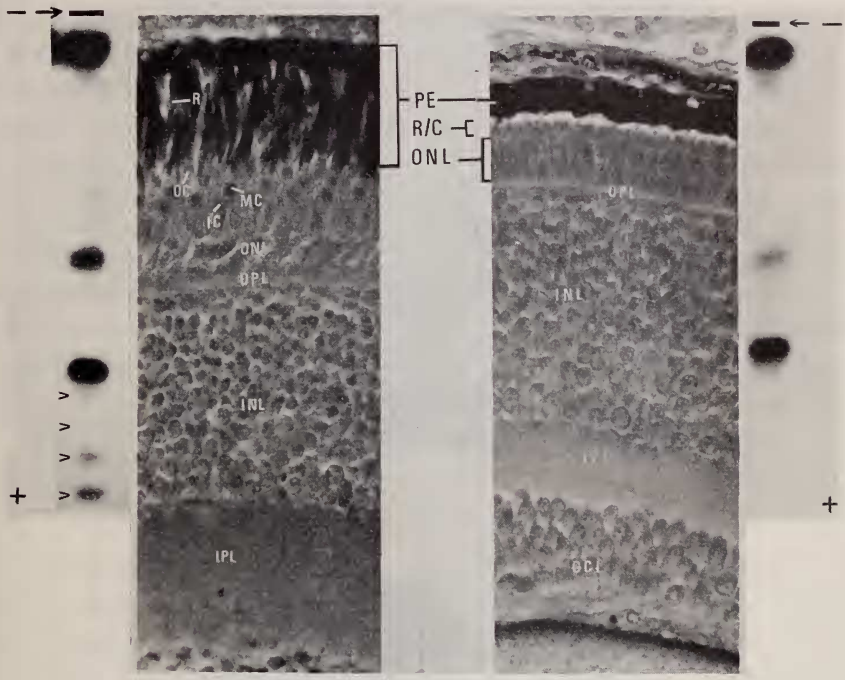

FIG. 13.

Lactate dehydrogenase isozymes of Lebistes eye at stage 7 (20th embryonic day) showing presence of E-bands.

FIG. 14.

Retina of stage 7, with differentiated photoreceptors and retinomotor response (azan stain).

FIG. 15.

Retina of stage 6, Photoreceptors present, but not yet differentiated (azan stain).

FIG. 16.

Lactate dehydrogenase isozymes of *Lebistes* eye at stage 6 (15th embryonic day). No E-bands present.

all three types of cones and the rods are differentiated at this stage (fig. 14). As a comparison, stage 6 (15th day) is shown; at this stage the photoreceptors are present but not yet differentiated, and the E-bands are not yet resolved (figs. 15 and 16). LDH staining of the kryostat sections of stage 7 shows a

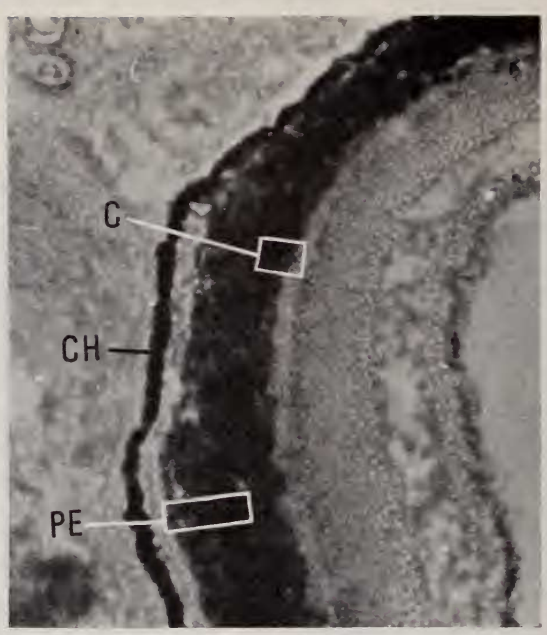

FIG. 17.
Retina of *Lebistes* (stage 7, 20th embryonic day) stained for Lactate dehydrogenase activity.

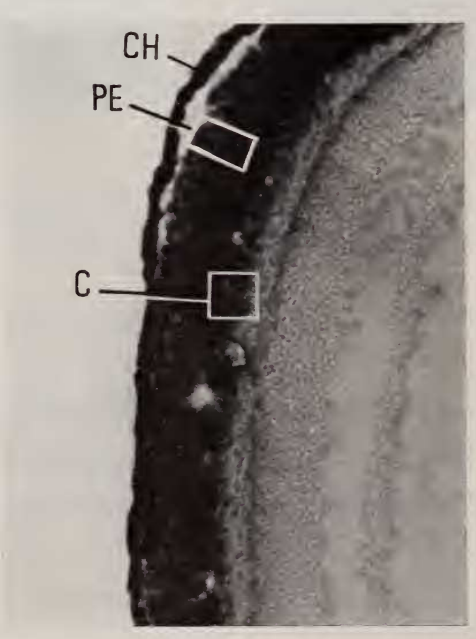

FIG. 18.
Same as fig. 17, but treated with inhibitor to show E-isozymes (incubation mixture see fig. 5).

preponderance of enzyme activity, as well as of E-isozyme activity, in the photoreceptor layer (figs. 17 and 18). When viewed with oil immersion, it can be readily seen that the formazan deposits of sections treated both with and without inhibitor, are located predominantly in the outer and inner segments of all three types of cones. It can be further observed that the outer segments of the rods contain pink stain in the inner, and blue granules in the outer half. Parallel experiments with 1) acetone extraction previous to incubation and 2) without lactate in the incubation mixture, again suggests that the blue droplets represent formazan absorbed by lipid material and that the pink stain is due to " nothing dehydrogenase ". Thus, the results indicate that the differentiated eye (stage 7) of *Lebistes* has the same LDH and E-isozyme distribution as the adult eye.

The E-isozyme bands on starch gel, and the E-isozyme distribution shown by kryostat sections, once established (stage 7), do not change during the remaining embryonic, post-embryonic and growth phases. The only noticeable difference is seen in the generally weaker expression of the E-isozymes in the embryonic phase.

Discussion

The distribution of LDH has been studied in the eye of the rabbit and the monkey (Graymore and Kissun, 1969; Lowry *et al.*, 1956). In the rabbit, the photoreceptors stain up very weakly, or not at all, whereas the inner layers exhibit more LDH activity. In the inner layers of monkey retina, there is less LDH activity than in rabbit retina (fig. 19). Lowry *et al.* explain this by the difference in blood supply in the two species: the monkey has two sets of vessels, i.e. one in the choroid and another one on the inner surface of the retina. The latter sends capillaries as far as the inner nuclear layer. In the rabbit, however, this inner set of vessels is missing over most of the retina. The authors assume that a glycolytic metabolism (LDH) might therefore be required for the inner layers in the rabbit. The blood supply to the eye of *Lebistes* is furnished almost exclusively by the choroid vessels. There can be seen a few capillaries along the inner surface of the retina, but they do not penetrate it. This condition resembles that of the rabbit eye. However, the LDH activity is most intense in the outer regions of the *Lebistes* eye, that is, nearest to the choroid blood supply.

The difference between the LDH activity of rabbit and monkey photoreceptors, reported by Lowry *et al.* (1956), is difficult to interpret. The rabbit has only rods in the retina, whereas in the monkey it is made up of both rods and cones; yet they were not analyzed separately. Thus, the fact that LDH activity is higher in the photoreceptors of the monkey may suggest that in mammals LDH is present predominantly in the cones, as is the case in *Lebistes*.

LDH activity has been investigated in photoreceptors of other mammalian species, of birds and of the frog. The results are not included in this comparison since the respective workers did not include phenazine methosulphate (PMS) in the incubating medium of their kryostat sections. When this electron carrier is

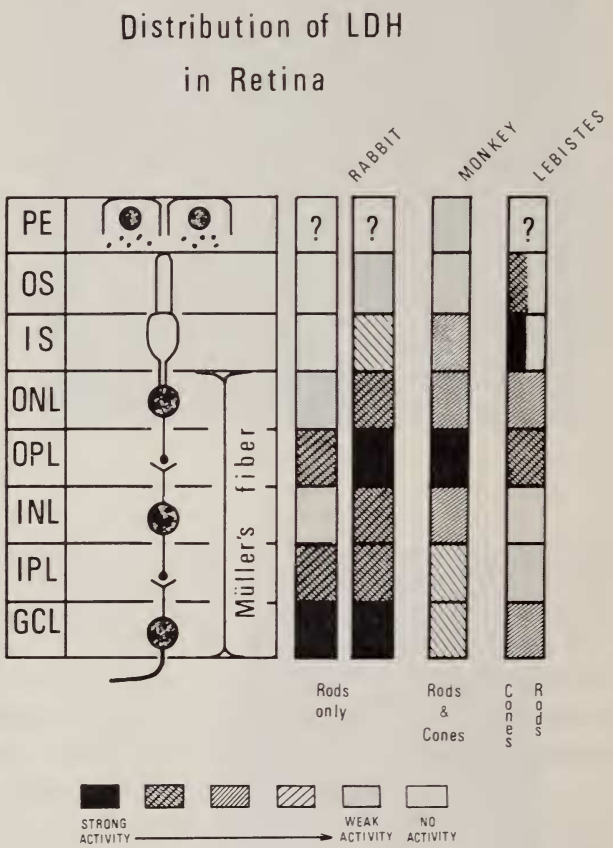

FIG. 19.

Rabbit, left column = diagram based on histochemical results by GRAYMORE and KISSUN 1969.
Rabbit, right column; monkey = diagrams based on biochemical results by LOWRY et al. 1956.

omitted, the sites of diaphorase activity (and not of dehydrogenase activity) are stained with formazan (GRAYMORE and KISSUN, 1969).

Indirect biochemical approaches, comparing intact retinae with retinae lacking photoreceptors, indicate that the photoreceptors of rabbit and rat possess unusually high respiration and glycolysis (COHEN and NOELL, 1965). These authors suggest a compartmentation of respiration and glycolysis within the

photoreceptor. They claim that this is supported by histochemical experiments showing the complementary subcellular distribution of enzymes required for glucose oxidation, and LDH, which is required for glycolysis. LDH is most abundant in the regions of the cell which have no mitochondria (LOWRY *et al.*, 1956). In *Lebistes*, however, LDH activity is evenly distributed over the whole inner segment.

In the retina of *Lebistes*, most of the LDH activity, and in particular, all of the E-isozyme activity, resides in the cones. The first differentiation of the photoreceptors in the embryo is simultaneous, i.e. all three types of cones, and the rods, are formed simultaneously. The structure of the newly formed cones and rods, as revealed by ordinary histological methods, is the same as in the adult eye. (MÜLLER, 1952). This seems to be reflected in the pattern of the E-bands: all bands appear simultaneously, at stage 7, and show already adult distribution of intensity. All through the growth phase of the retina, new photoreceptors are being formed at the periphery. Kryostat sections of all stages show always intense activity of E-isozymes also in these newly differentiated cones.

When sections are treated with the inhibitor, formazan deposits in the cones are less intense. This would suggest that other isozymes than E-isozymes reside in the cones.

Perhaps the restriction of the E-isozymes to the cones in *Lebistes* is related to the unique tiered arrangement of the cones, which as such demands a functional explanation. It has been suggested, but not confirmed, that this arrangement should minimize, or even completely correct, the chromatic aberration; this would result in a greatly increased acuity of the eye (EBERLE, 1968). On the other hand, a double row of cones has been observed in two tree squirrels (*Sciurus carolinensis leucotis* and *Tamiosciurus hudsonicus loquax*), which show a pure cone retina. (TANSLEY, 1961). The author suggests that this arrangement may be a device to increase the sensitivity by increasing the number of photoreceptors converging on to each ganglion cell; this would, however, be expected to result in decreased visual acuity.

One other teleost, *Xiphophorus helleri*, has been tested for LDH (and E-isozyme) distribution in the eye (WHITT and BOOTH, 1970). The E-isozymes were found predominantly in the inner segments of the photoreceptor cell and in the outer nuclear layer. The authors fail, however, to differentiate between rods and cones. Both *Xiphophorus* and *Lebistes* belong to the *poeciliidae*. Judging from fig. 2 of their publication, the retina of *Xiphophorus* shows the same unusual pattern of cone distribution as *Lebistes*. Thus the heavy formazan deposits attributed by WHITT and BOOTH to the outer nuclear layer, are probably located in the ellipsoids of the inner cones. This assumption is based on the fact that in *Lebistes* the nuclei of the middle cones penetrate the outer limiting membrane, so that the ellipsoids of the inner cones seem to lie within the outer nuclear

layer. LDH activity in the Müller's fibres, as reported for *Xiphophorus*, is not evident in *Lebistes*.

WHITT (1970) has studied extensively the kinetic, physical and immunochemical properties of the E_4 isozyme (most anodic E band). He suggests that it may be specially suited for cells, such as the photoreceptors, with a high constant aerobic metabolism. He also discusses a working hypothesis that the E_4 isozyme plays an important role in the regeneration of rhodopsin in the photoreceptor of the teleost retina.

While evidence cannot be presented here concerning the degree of aerobic metabolism in *Lebistes*, it would seem that, if the above hypothesis applies to the cones, an alternative pathway must be operative for rhodopsin regeneration in the rods of *Lebistes*. Histochemical studies of a "normal" mixed, a pure rod, and a pure cone teleostean retina may shed further light on the differences between metabolism of rod and cone and on the significance of E-isozymes in particular.

ZUSAMMENFASSUNG

Das mit Stärkegel-Elektrophorese erhaltene Laktatdehydrogenase (LDH)-Muster des Auges von *Lebistes reticulatus* weist die für viele Teleostieraugen charakteristischen rasch wandernden E-Isozyme auf.

Histochemische Färbung von Gefrierschnitten zeigt, dass die Hauptaktivität der LDH in den Zapfen (Aussen-, Mittel- und Innenzapfen) liegt. In den Stäbchen-Aussengliedern konnte keine LDH-Aktivität, nur sogenannte "Nothing Dehydrogenase"-Aktivität, nachgewiesen werden. In den Stäbchen-Innengliedern war die LDH-Reaktion äusserst schwach im dunkeladaptierten Auge. Im helladaptierten Auge sind die Innenglieder nicht sichtbar, da im Pigment eingebettet.

Durch Zusatz eines Hemmstoffes (Harnstoff) kann die Aktivität der E-Isozyme allein dargestellt werden. Es zeigt sich, dass sich in der Photorezeptorenschicht nur die Zapfen anfärben: Die Innenglieder reagieren am stärksten, gefolgt von den Aussengliedern. Die äussere Kernschicht und die äussere Faserschicht reagieren nur schwach.

Während der Entwicklung treten die E-Isozyme erstmals im Auge des 20-tägigen Embryos (Embryonalperiode 30 Tage) auf. Elektrophoretisches Verteilungsmuster und Bandenzahl sind dieselben wie im Adultauge. Auch das histochemische Bild ist dasselbe wie in der adulten Retina.

Während der restlichen Embryonal-, der Postembryonal- und der Wachstumsperiode konnten keine Veränderungen des E-Isozym-Musters beobachtet werden.

Résumé

Le motif de Lactatdéhydrogénase (LDH) de l'œil de *Lebistes reticulatus* btenu par électrophorèse sur gel d'amidon présente les E-isozymes à migration apide caractéristiques des yeux de nombreux Teléostéens. La coloration histo-himique de coupes à la congélation montre que l'activité principale de la LDH lieu dans les cônes (extérieures, moyens et intérieurs). Dans le segment xtérieur des bâtonnets il n'a pas été mis en évidence d'activité LDH, mais eulement une activité sans LDH. Dans le segment intérieur des bâtonnets, a réaction LDH est extrêmement faible dans les yeux adaptés à l'obscurité. Dans les yeux adaptés à la lumière, les segments intérieurs ne sont pas visibles cause du pigment qui les enrobe.

Par l'addition d'un inhibiteur (urée) on peut montrer l'activité des E-isozymes euls. Les cônes seuls se colorent, les segments intérieurs réagissant le plus vigoureusement, suivis par les segments extérieurs. La couche de noyaux externe et a couche de fibres externe ne réagissent que faiblement.

Pendant le développement, les E-isozymes apparaissent dans l'œil de l'embryon le 20 jours (sur une période embryonnaire de 30 jours). La répartition électrophorétique et le nombre de bandes sont les mêmes que chez l'adulte. Aucun hangement du motif des E-isozymes n'a été observé non plus pendant le reste lu développement.

LIST OF ABBREVIATIONS

C	Cones
CH	Choroid
GCL	Ganglion cell layer
IC	Inner cone
INL	Inner nuclear layer
IPL	Inner plexiform layer
IS	Inner segment
MC	Middle cone
OC	Outer cone
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
PE	Pigment epithelium
R	Rods

BIBLIOGRAPHY

Batellino, L. J., F. R. Jaime and A. Blanco. 1968. *Kinetic properties of rabbit testicular lactate dehydrogenase isozyme.* J. Biol. Chem. 243: 5185-5192.

Cohen, L. H. and W. K. Noell. 1965. *Relationships between visual function and metabolism.* In " Biochemistry of the retina ", Acad. Press Inc., London.

GRAYMORE, C. N. and R. D. KISSUN. 1969. *Use of phenazine methosulphate (PMS) in th histochemical localization of lactic acid dehydrogenase (LDH) in th retina*. Exptl. Eye Res. 8: 375-378.

EBERLE, H. 1968. *Zapfenbau, Zapfenlänge und chromatische Aberration im Auge vo Lebistes reticulatus Peters (Guppy)*. Zool. Jb. Physiol. 74: 121-154.

KUNZ, Y. 1963. *Die embryonale Harnblase von Lebistes reticulatus*. Rev. suisse Zool. 70 291-297.

— 1964. *Morphologische Studien über die embryonale und postembryonale Entwicklun bei Teleostiern mit besonderer Berücksichtigung des Dottersystems un der Leber*. Rev. suisse Zool. 71: 445-525.

— 1971. *Dorsal headfolds in the embryo of the viviparous teleost Lebistes reticulatus* Rev. suisse Zool., 78: 187-207.

LOWRY, O. H., N. R. ROBERTS and C. LEWIS. 1956. *The quantitative histochemistry o the retina*. J. Biol. Chem. 220: 879-892.

MARKERT, C. L. and I. FAULHABER. 1965. *Lactate dehydrogenase isozyme patterns o fish*. J. Exp. Zool. 159: 319-332.

MASSARO, E. J. and C. L. MARKERT. 1968. *Isozyme patterns of salmonid fishes : Evidenc for multiple cistrons for lactate dehydrogenase polypeptides*. J. Exp Zool. 168: 223-238.

MÜLLER, H. 1952. *Bau und Wachstum der Netzhaut des Guppy (Lebistes reticulatus)* Zool. Jb. (Phys.) 63: 275-324.

TANSLEY, K. 1961. *Comparative anatomy of the mammalian retina with respect to th electroretinographic response to light*. In: The structure of the eye Acad. Press., N.Y. and London.

WHITT, G. S. and G. M. BOOTH. 1970. *Localization of lactate dehydrogenase activity i the cells of the fish (Xiphophorus helleri) eye*. J. exp. Zool. 174: 215-224

WHITT, G. S. 1970. *Developmental genetics of the lactate dehydrogenase isozymes of fish* J. exp. Zool. 175: 1-36.